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A Possible Sugar Receptor Protein Found in the Labellum of the Blowfly, *Phormia regina*

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ABSTRACT—Previous physiological studies have suggested the existence of at least three functionally separated receptor sites in the labellar sugar receptor of the fly, called the pyranose (P site), the furanose (F site) and the third sites (T site), and that starch acts as a competitive inhbitor for the P site. I detected in this work a new candidate protein for the P site in the labellar extract by affinity electrophoresis with starch. The dissociation constant of the candidate protein-starch complex was estimated to be 0.7%, a value consistent with the electrophysiological estimate of the inhibition constant for starch on the sugar response. The stimulus sugars for the P site bound to the candidate protein in competition with starch. The dissociation constants of the candidate protein-sugar complexes were highly correlated with the electrophysiological constants defined as the sugar concentrations which give rise to half maximal responses. However, the stimulus sugars for the F site did not compete with starch for the candidate protein. The candidate protein was water insoluble and appeared to be located in the distal process and the cell body, but not in the axon, of the labellar chemosensory cell.

INTRODUCTION

Since the pioneering work of Dastoli and Price [1], repeated attempts have been made to identify sweet-taste receptor molecules in both vertebrates and invertebrates. However, no taste receptor proteins have been definitively identified yet, and biochemical studies on the sense of taste have met with only limited success.

In the fly, many behavioral and electrophysiological studies have suggested that the sugar receptor cell is sensitive to a broad spectrum of chemicals and that it has multiple receptor sites. At least three functionally separate receptor sites have been documented in the sugar receptor cell of the fleshfly [2–4]. They are the pyranose site (P site) sensitive to sucrose, maltose, D-glucose, L-fucose, etc., the furanose site (F site) sensitive to D-fructose, D-fucose, etc., and the third site (T site) sensitive to aliphatic carboxylate anions. As a working hypothesis, the P site was proposed to be identical with an α-glucosidase [5], but some inconsistencies have been reported for the hypothesis [6]. I recently found that starch acts as a competitive inhibitor on the P site [7]*. Therefore, it was possible to detect a new candidate protein for the P site by affinity electrophoresis with starch. In this paper, I report on the identification of the candidate protein based on its affinity for starch or stimulus sugars and its location in the chemosensory cell.

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MATERIALS AND METHODS

Sample preparation

Five to seven-day-old blowflies, *Phormia regina*, reared at 20–25°C and fed with 0.1 M sucrose were used. Living flies were anesthesized by cooling on ice, and the labella were cut at the distal end of the proboscis. Two hundred labella so obtained were frozen with a small volume of liquid nitrogen in a hand mortar. After the frozen labella were homogenized for 20 min, the homogenate was suspended in 0.1 ml of sample buffer (4.65 mM sodium barbiturate-HCl, 2% Triton X-100, 10%

* Paper presented by the author in her maiden name, Hara.

glycerol, pH 6.8) and incubated at 4°C for 1 hr. The suspension was centrifuged at 3,000 rpm at 4°C for 10 min, and the supernatant was used as the sample extract. The extract of the proboscis without labellum was prepared in the same way to serve as control. To examine the water soluble fraction, the labellar homogenate was suspended in distilled water, incubated at 4°C for 1 hr, and centrifuged at 50,000 rpm at 4°C for 1 hr. The supernatant was then lyophilized and dissolved in the same buffer.

In addition, the labellum was separated into "labellar content" and the "labellar integument" following the method of Amakawa *et al.* [8]. Labella were gently sonicated, 150–200 at a time, in 5 ml of distilled water with a Tominaga model UR-150P ultrasonicator (25 kw output) on ice for 5 min. Subsequently, 500 "labellar integuments", or cuticle lobes, were collected and washed twice with distilled water. The extract was prepared in the same way as the whole labellum extract. The "labellar contents", which were washed out from the 500 labella into distilled water during sonication, were collected by lyophilization. They were then homogenized with 0.1 ml of sample buffer in a glass homogenizer on ice for 10 min and incubated at 4°C for 1 hr. The supernatant obtained following centrifugation at 3,000 rpm for 10 min was used as the sample extract.

Electron microscopy

For the scanning electron microscopy, whole labella or "labellar integuments" were fixed in 3% glutaraldehyde at 20°C for 1 hr, dehydrated through ethanol series and isoamylacetate, dried at the critical point of CO_2 with a Hitachi model HPC-2 dryer, and coated with gold by spattering with an Eiko model IB-3 ion-coater. They were then observed with Hitachi model S-430 scanning electron microscope.

For the transmission electron microscopy, whole labella or "labellar integuments" were fixed in 3% glutaraldehyde at 20°C for 1 hr and 2% osmium tetroxide on ice for 1 hr, dehydrated through ethanol series and propylene oxide, and embedded in Epon 812 resin. Sections were cut with a Porter-Blum model MT-1 ultramicrotome, double-stained with lead acetate and uranyl acetate and observed with a Hitachi model H-300 electron microscope.

Affinity electrophoresis

As many differnt kinds of proteins are present in the blowfly labellum, it was difficult to detect a minor protein such as the sugar receptor protein in one-dimensional electrophoresis. Therefore, two-dimensional polyacrylamide gel electrophoresis, in which an affinity ligand was added to the running gel in the first dimension, was adopted. The gel system was similar to the Ornstein and Davis's stacking system [9, 10], except that Triton X-100 (2% at the final concentration) was added to the stacking (4.5% acrylamide) and running gels (7.5% acrylamide), and barbiturate buffers (stacking gel buffer: 9.3 mM sodium barbiturate-HCl, pH 6.7; running buffer: 91.1 mM sodium barbiturate-HCl, pH 8.9; electrode buffer: 41.1 mM sodium barbiturate-glycine, pH 8.3) were used instead of Tris buffers, which inhibit sugar responses [11]. Proteins were detected by the silver staining method of Oakley *et al.* [12]. The first dimensional electrophoresis was carried out with 10–20 μl of the sample extract at 3 mA for 100 min in a disc gel (2 mm in diameter, 130 mm in length). The gel was removed into sample buffer, shaken at room temperature (20–25°C) for 1 hr, and loaded onto a slab gel ($130\times115\times1$ mm) for the second dimensional electrophoresis, carried out at 30 mA for 250 min. The composition of gels and buffers used in the two electrophoretic runs were similar except for the starch added in the first dimension. To evaluate the affinity of sugars for the candidate protein, stimulus sugar was added to the running gel, together with starch, in the first dimensional electrophoresis. During electrophoreses, the gel temperature measured through the glass tube or plate was 21 ± 2°C, and the pH of the running gel was 9.4 immediately after the run.

Calculation of dissociation constant

Starch is a large polysaccharide molecule with no electric charge, which, when complexed with a protein, greatly retards the mobility of the protein during electrophoresis. Therefore, if the mobility of the protein, m_o, decreases to m in the presence of starch of concentration $[I]$, the protein-starch

interaction can be expressed by the following equation [13]:

$$m_o/m = 1 + [I]/K_i \qquad (1)$$

where K_i is the dissociation constant of protein-starch complex. Thus, the plot of m_o/m against $[I]$ yields a straight line with $[I]$ intercept at $-K_i$. Here the dissociation constant is expressed in % w/v because starch is polydisperse and therefore does not lend itself to the molarity measure.

On the other hand, stimulus sugars for the sugar receptor are much smaller than starch and have no electric charge so that the mobility of the protein-sugar complex is nearly the same as that of the free protein. Therefore, if the mobility, m, of a protein in the presence of starch becomes m' in the presence of both sugar (concentration, $[S]$) and starch (concentration, $[I]$), the protein-sugar interaction is given by the following equation [14]:

$$m'/(m_o - m') = K_i(1 + [S]/K_d)/[I] \qquad (2)$$

where K_d is the dissociation constant of protein-sugar complex. Thus, the plot of $m'/(m_o - m')$ against $[S]$ gives a straight line intercepting the $[S]$ axis at $-K_d$. From Eqs. (1) and (2) it may be seen that if m' is equal to m, K_d is infinitely large. This means that the protein-sugar interaction through the starch binding center is negligible. That is, the protein migrates as if the gel contained only starch.

Assay of α-glucosidase

The α-glucosidase activity in the disc gel was assayed with *p*-nitrophenyl α-D-glucopyranoside (α-PNPG) [15]. After the electrophoresis in the first dimension, the disc gel was removed from the glass tube, and the running gel part was divided equally into 19 pieces (5 mm length). Each piece was incubated in the reaction mixture containing 10 mM α-PNPG in 0.5 ml of 0.1 M sodium citrate buffer (pH 6.0). After incubation at 27°C for 1 hr, the reaction was stopped by adding 2 ml of 0.5 M Tris-HCl (pH 9.0), and the absorbance of liberated *p*-nitrophenol was measured at 410 nm with the Shimadzu model UV-202 recording spectrophotometer.

Chemicals

Sucrose, maltose, D-glucose, D-xylose, L-sorbose and D-fructose were purchased from Wako Pure Chemicals, Osaka, Japan. D- and L-fucose were obtained from Nakarai Chemicals, Ltd., Osaka Japan.

RESULTS

Affinity for starch

When two-dimensional electrophoresis of the labellar extract was carried out without starch, all proteins whose mobilities are different from each other migrated into a diagonal line on the slab gel, because each individual protein in the labellar extract showed the same mobility in the first dimension as in the second (Fig. 1a). When the electrophoresis was carried out with starch in the first dimension, however, a single spot was reproducibly found separated from the diagonal line. Figure 1b, c and d show that the mobility of this protein in the first dimensiom decreases with increasing concentration of starch in the running gel. In this way, a protein with affinity for starch was easily detected using this system. The values of m_o and m for the protein were directly measured on each gel (see Fig. 2b), and the ratio of mobilities, m_o/m, was plotted against the concentration of starch, $[I]$, to estimate the dissociation constant of the protein-starch complex, K_i, to be 0.7% (Fig. 2). The value is consistent with an electrophysiological estimate of the inhibition constant for starch at the P site of the sugar receptor at 22±2°C, i.e. around 0.6% [7]. The responsiveness of the fly to sugars is stable over a pH range of 3 to 10 [16]. Thus, these electrophoretical and electrophysiological estimates are comparable with each other.

Affinity for sugars

L- and D-fucose stimulate the P and the F sites, respectively [2], though they are neither metabolized in the blowfly [17] nor bind to the membrane-bound α-glucosidase in the blowfly labellum [6]. When electrophoresis was carried out in the presence of both D-fucose and starch, a protein spot was detected almost in the same position (Fig. 3c) as in the presence of starch alone (Fig. 3a). When L-fucose was added instead of D-fucose,

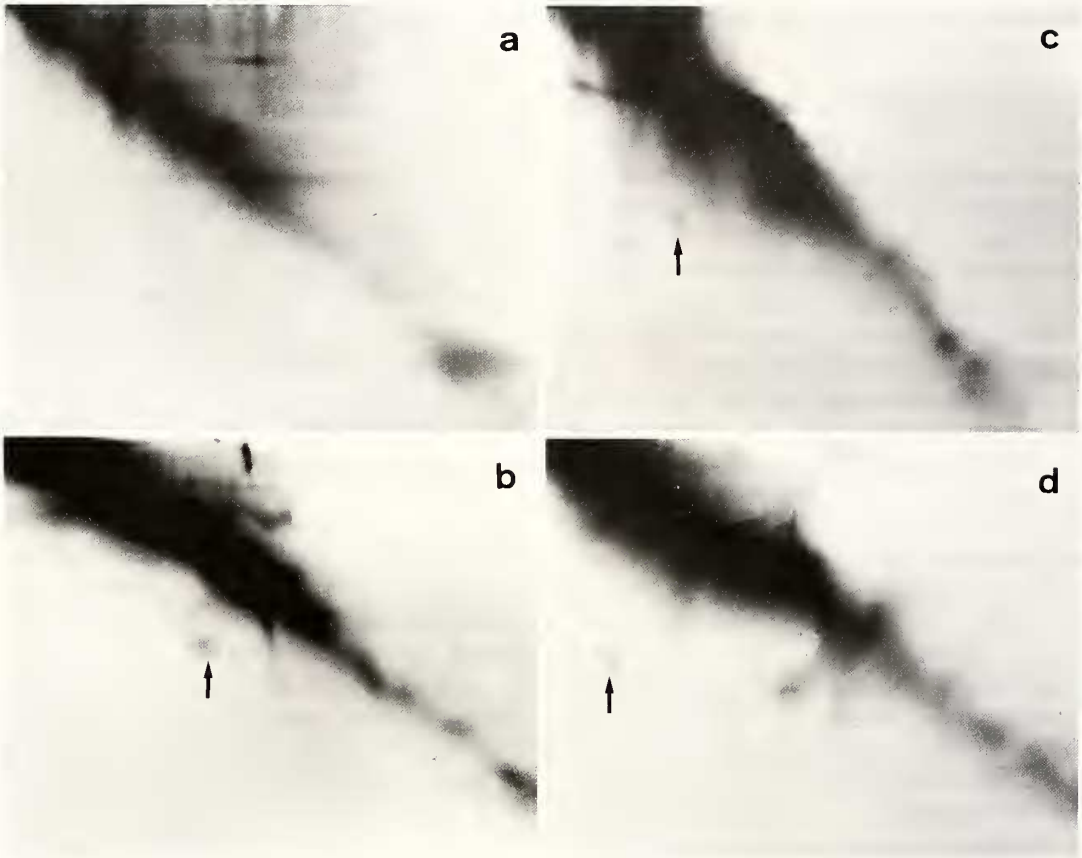

FIG. 1. Two dimensional affinity electrophoresis of the labellar extract: (a), 0%; (b), 0.5%; (c), 1%; (d), 2% starch in the first dimension. In Figs. 1 and 3, the origin of electrophoresis is the upper left-hand corner, and the proteins migrated toward the right and the bottom, respectively, in the first and the second dimensional electrophoreses (see Fig. 2b). The candidate protein spot is indicated by an arrow in each figure.

however, this spot was detected closer to the diagonal line (Fig. 3b). These results indicated that D-fucose did not compete with starch for the protein but L-fucose did. Thus, this protein is a possible sugar receptor molecule for the P site but not for the F site. Moreover, this new candidate protein very likely is different from α-glucosidase, since L-fucose does not bind to α-glucosidase [6]. Figure 4 shows the plot of the ratio $m'/(m_o-m')$ against the concentration $[S]$ of L- (*a*) and D-fucose (*b*), respectively. The calculated dissociation constant of the candidate protein-L-fucose complex was 254 mM, while that of the candidate protein-D-fucose complex was infinite.

Some other stimulus sugars for the P site were also examined, and the dissociation constants of the complexes between the candidate protein and these sugars are listed in Table 1. These dissociation constants were calculated using Eq. (2) in Materials and Methods. For sucrose, maltose, D-glucose and D-fructose, the dissociation constants, K_d, were compared with the mid-point concentrations, K_b, defined as the concentration of stimulus which gives rise to half maximal electrophysiological responses. As seen in Table 1, the dissociation constant was 4–5 fold larger than the mid-point concentration for sucrose, maltose or D-glucose but infinitely larger than that for D-fructose.

Localization of the candidate protein

The water soluble fraction of labellum was

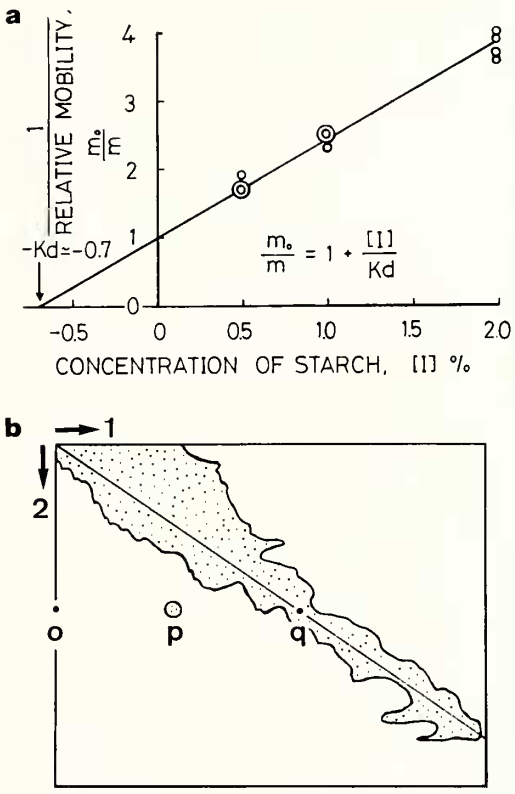

FIG. 2. (a) Determination of the dissociation constant of the candidate protein-starch complex by plotting the reciprocal of the relative mobility of the candidate protein, m_o/m, against the concentration of starch, $[I]$. (b) Illustration for the measurement of m and m_o. In the presence of starch, the candidate protein migrates to the position p. The position to which it would have migrated in the absence of starch, q, is estimated by extending the line op horizontally to the diagonal line defined by the protein stain (dotted area). When the protein stain appeared smeared in the central portion, the diagonal line was defined as that line from the origin of the electrophoresis to some distinguishable spots near the leading front of protein migration. The mobilities, m and m_o, are proportional to the distance op and oq, respectively.

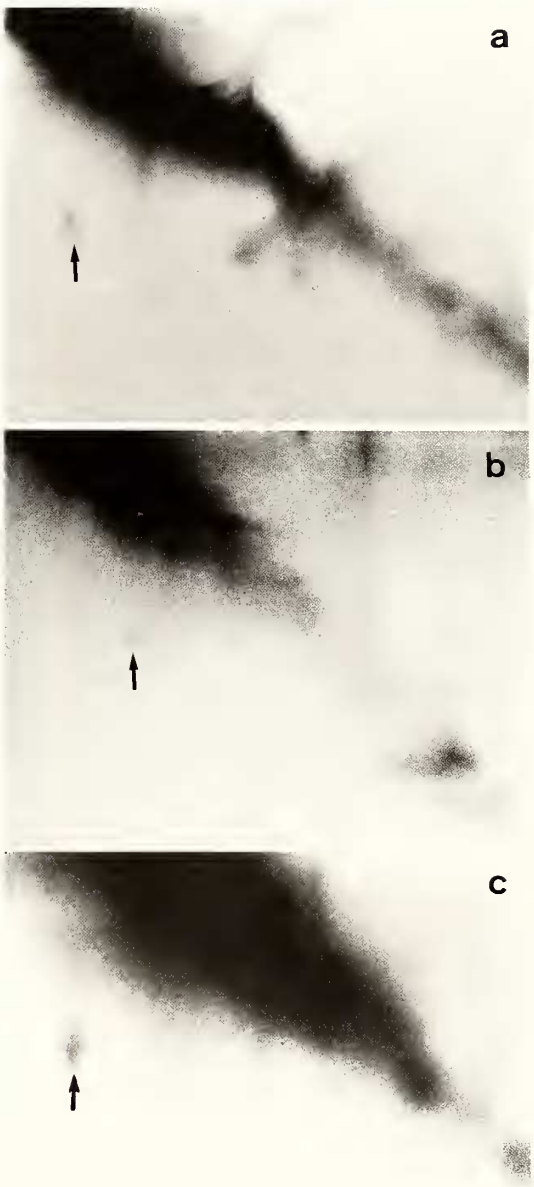

FIG. 3. Comparison of affinity for the candidate protein between L- and D-fucose. (a) same electrophoretic pattern shown in Fig. 1d presented as no sugar control; (b) 0.2 M L-fucose plus 2% starch; (c) 0.2 M D-fucose plus 2% starch in the first dimension.

examined first for the presence of the candidate protein, but the candidate protein was not detected. The extract of the proboscis from which the labellum was removed also yielded negative results. These results suggested that the candidate protein was a labellum specific, membrane-bound protein. I attempted to determine in what part of

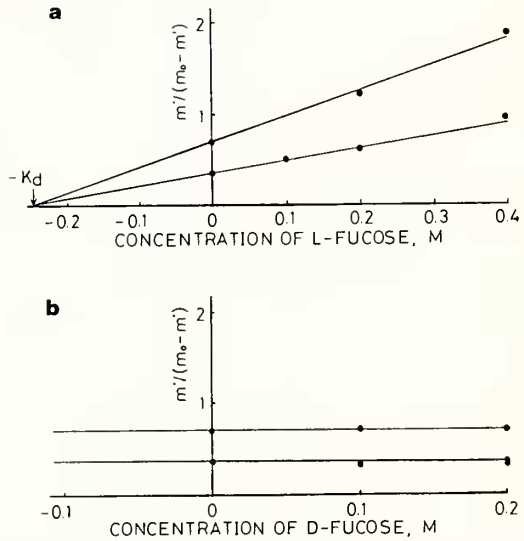

FIG. 4. Determination of the dissociation constant of the candidate protein-sugar complex by plotting $m'/(m_o-m')$ against the concentration of L-fucose (a) and D-fucose (b).

the sensory cell the candidate protein is located. Although the sensillum tip is especially rich in receptor membranes, they are too thin to cut and to collect. Therefore, I attempted to isolate the receptor membranes by sonication. Sonication separated the "labellar integument", which contained the receptor membranes, from the "labellar content", which consisted of the sensory cell bodies, labial nerve, supportive cells and connective tissues. In the intact labellum, the cell bodies of 4 chemosensory and a mechanosensory cells are surrounded by supportive cells at the base of each chemosensillum, and their axons extend into the labial nerve. In the "labellar integument", however, all these structures were completely removed, exposing the inside surface of the cuticle (Fig. 5a, c). Nevertheless, the chemosensilla were still attached to "labellar integument" preserving the membrane fragments in the inner lumen (Fig. 5d). These membrane fragments were thought to be derived from the distal processes of the chemosensory cells. In both the "labellar content" and the "labellar integument", the candidate protein was detected as a spot separated from the diagonal line, similar to the spot seen in Figure 1. Thus, the candidate protein seemed to be located in both the cell body and the distal process of the sensory cell.

α-Glucosidase

To examine the hypothesis that an α-glucosidase is the P site molecule, I compared the mobility of the α-glucosidase with that of the newly detected candidate protein in disc gel electrophoresis in the presence of varying concentration of starch. The α-glucosidase activity was always found at 30–35 mm from the origin regardless of the presence of starch, while the mobility of the new candidate protein decreased with increasing concentrations of starch (Fig. 6).

TABLE 1. Dissociation constant of candiate protein-sugar complex

Stimulu sugar	Site specificity[1)]	$K_d \pm$S.D. (mM)	Test No.	$K_b \pm$S.D.[2)] (mM)	Test No.[2)]
sucrose	P	104±25	4	21±10	25
maltose	P	110±20	2	26±6	15
L-fucose	P	254±21	5		
D-glucose	P	360±25	4	83±27	8
L-sorbose	P	575±98	3		
D-xylose	P	1504±596	2		
D-fructose	F	∞	4	53±17	14
D-fucose	F	∞	5		

1): Shimada (1974), 2): Hara (1983)
∞, Calculated value is more than 10 M.

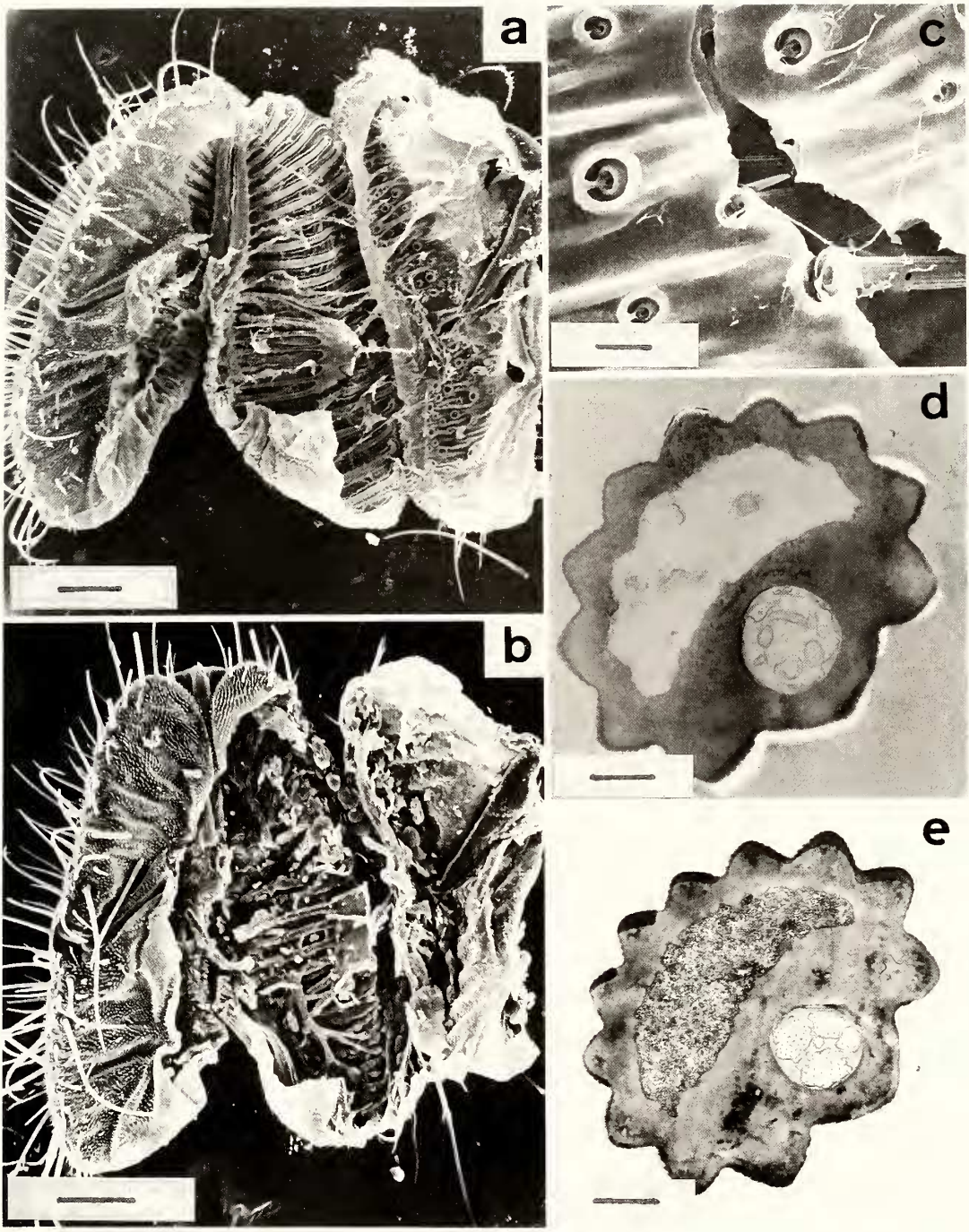

FIG. 5. Electron micrographs of "labellar integument" and intact labellum. (a) and (b), scanning electron micrographs of the internal appearance of labellar lobes of a "labellar integuments" and an intact labellum, respectively; (c), scanning electron micrograph of the bases of chemosensilla seen from the inside of a "labellar integument". An attached sensillum can be seen through the crack in the cuticle; (d) and (e), cross sections of chemosensilla of the "labellar integument" and the intact labellum, respectively. Bars indicate 100 μm (a, b), 10 μm (c), and 1 μm (d, e).

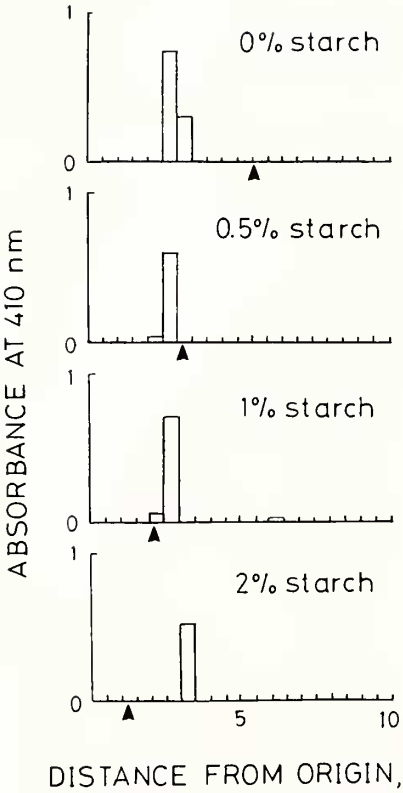

FIG. 6. Localization of α-glucosidase activity in the disc gel used in the first dimension under varying concentrations of starch. The ordinate indicates the α-glucosidase activity determined by the absorbance at 410 nm, and the abscissa shows the distance from the origin in the running gel. The location of the candidate protein in the disc gel was estimated by two dimensional electrophoresis (arrow heads).

DISCUSSION

Affinity electrophoresis

It has been thought that, in the taste receptor, the receptor-stimulus interaction is too weak and the quantity of the receptor protein is too small to detect or isolate the receptor protein. In the labellar sugar receptor of the fly, the P site-sucrose interaction is comparatively strong, but the dissociation consant of the P site-sucrose complex is still more than 0.01 M, according to Morita [18]. Such a weak interaction cannot be detected directly by any means other than affinity electrophoresis. For the application of affinity electrophoresis, however, it is imperative that affinity ligand is water-soluble and immobile in the polyacrylamide gel. Starch becomes water-soluble when heated. Its molecular weight, estimated to be 10–100 times larger than that of the candidate protein, was expected to be large enough for it to be immobile in 7.5% polyacrylamide gel. The result shown in Figure 2 satisfied the linear relationship between m_o/m and $[I]$ demanded by Eq. (1), strongly suggesting that starch when complexed with the candidate protein was indeed immobile in the polyacrylamide gel. Thus, starch is a satisfactory affinity ligand and has already been used in affinity electrophoresis for phosphorylase [13, 14] or amylase [19]. As in the experiments on phosphorylase or amylase, the P site-starch interaction was strong enough to detect the P site molecule. Furthermore, the two dimensional electrophoresis technique applied here was very useful in isolating the protein of interest among many different kinds of protein.

As for the quantity of the receptor protein, Hansen and Wieczorek [20], on the basis of semi-quantitative calculation, estimated that 10^{-7} or 10^{-10} g of labellar sugar receptor protein can be obtained from 1,000 flies. The silver staining method of Oakley *et al.* [12] is sufficiently sensitive to detect protein density of 10^{-11}–10^{-10} g/mm^2 on a gel plate. Since the candidate protein extracted from 20–40 flies made a visible spot about 2 mm diameter on the gel, at least 10^{-9} g of the candidate protein should be obtainable from 1,000 flies, consistent with the estimate of Hansen and Wieczorek [20].

Receptor-sugar interaction

The receptor-stimulus interaction is thought to be the primary process in the chemosensory transduction mechanism. The existence of the specific receptor molecule has not always been accepted in the case of salt, water or bitter taste reception [21]. In the case of sugar or amino acid reception, however, it is generally accepted that a specific receptor molecule mediates the receptor function [20, 21]. The electrophysiological analysis of the sugar receptor cell of the fly, in particular, is well developed, and the relation between the dissociation constant of the receptor-sugar com-

plex, K_d, and the mid-point concentration, K_b, is described [18] as follows:

$$K_d = K_b(sg/G+1),$$

where s is the total receptor site; g, the conductance per activated site; G, the conductance across the receptor membrane in the resting state. Applying the electrophysiological data on the recovery process of the sucrose response to the above equation, Ninomiya *et al.* [23] obtained

$$sg/G = 4 \text{ or}$$
$$K_d = 5K_b .$$

This is in good agreement with my result, i.e., $K_d = 4K_b$ (Table 1). These results may be interpreted in terms of amplification at the conductance level. That is, in the primary process of the chemosensory transduction in the sugar receptor of the fly, sucrose, maltose, D-glucose, etc., bind to the P site molecule, consisting of the candidate protein, according to their individual affinities, but the conductance change across the sugar receptor membrane uniformly gives 4 to 5-fold amplification of the sensitivity.

Table 1 shows the calculated dissociation constants, assuming the simple case that one molecule of starch or sugar binds to the P site molecule. Therefore, the calculated value for D-glucose is probably an overestimate because two glucose molecules are thought to bind to each P site molecule [24].

Location of the sugar receptor protein

The candidate protein was found in the "labellar content" and the "labellar integument" but not in the proboscis from which the labellum had been cut off. This suggested that the candidate protein is located in the cell body and the distal process but not in the axon of the labellar chemosensory cell. The P site molecule is probably synthesized in the cell body of the sugar receptor cell, transported to the distal process and concentrated in the receptor region at the tip. Recently, we suggested that the P site is located not only at the tip but also in the intermediate length of the distal process [23] and that a considerable number of receptor molecules for the P site exist in regions other than the receptor region of the sugar receptor cell [25].

Comparison with α-glucosidase

Many behavioral and electrophysiological studies have documented the stereospecificity of the P site for stimulus sugar [2, 7, 26], and it has been suggested that three successive equatorial hydroxyl groups in the chair form of the pyranose ring are essential for stimulation at the P site regardless of their position. Actually, all sugars which bind to the candidate protein have this essential structure and are stimulatory at the P site. As for starch, its glucopyranose residues do not have this essential structure, but it can compete with the stimulus sugars for the P site [7]. Although all stimuli which have been estimated behaviorally or electrophysiologically could not be investigated in the present work, the candidate protein showed binding specificity for several sugars and starch similar to that exhibited by the P site in behavioral or electrophysiological studies. However, L-fucose and starch do not bind to the labellar α-glucosidase. Thus, I conclude that this candidate protein is different from the α-glucosidase, and that it is rather likely to be the P site receptor molecule.

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